Supporting Information

A Dendrimer Scaffold for the Amplification of In Vivo Pretargeting Ligations

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MATERIALS AND METHODS

Unless otherwise noted, all chemicals were acquired from Sigma-Aldrich (St. Louis, MO) and were used as received without further purification. All water employed was ultrapure (>18.2 $M\Omega cm^{-1}$at 25 °C), all DMSO was of molecular biology grade (>99.9%), and all other solvents were of highest grade commercially available. Amine-reactive *trans*-cyclooctene [(E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate; TCO-NHS)] and amine-reactive tetrazine (2,6-dioxo-1-pyrrolidinyl 5-[4-(1,2,4,5-tetrazin-3-yl)benzylamino]-5-oxopentanoate; Tz-NHS) were purchased form Sigma-Aldrich (St. Louis, MO), p-NCS-Bn-DFO and DiAmSar chelators were purchased from Macrocyclics (Dallas, TX), and [^{64}Cu]Cu-SarAr-Tz and Tz-PEG_7-AF680 were synthesized as previously reported.[1] Disulfide dendrimer (code AuCS-294) was purchased from Andrews ChemServices (Berrien Springs, MI). DBCO-PEG_4-Maleimide, and DBCO-PEG_{12}-TCO were purchased from Click Chemistry Tools (Scottsdale, AZ). Humanized A33 (huA33) was generously provided by the Ludwig Institute and stored at -80 °C prior to use. Remove-iT^{TM} EndoS enzyme was purchased from New England BioLabs (Ipswich, MA) and Gal-T(Y289L) purchased from ThermoFisher (Waltham, MA). ^{64}Cu was purchased from Washington University, St. Louis, where it was produced on a medical cyclotron (Model CS-15, Cyclotron Corporation) via the ^{64}Ni(p,n)^{64}Cu transformation and purified as previously described to yield [^{64}Cu]$CuCl_2$ with an effective specific activity of 518 ± 259 GBq/μmol (14,000 ± 7600 mCi/μmol).[2] ^{89}Zr was produced on a TR19/9 cyclotron (Ebco Industries Inc.) at Memorial Sloan Kettering Cancer Center via the ^{89}Y(p,n)^{89}Zr reaction and purified to yield ^{89}Zr-oxalate with a specific activity of 195-497 MBq/μg. Amine-reactive AlexaFluor 680 (AF680-NHS) was purchased from ThermoFisher Scientific (Waltham, MA). All experiments using laboratory animals were performed in accordance with protocols approved by the Memorial Sloan Kettering Institutional Animal Care and Use Committee.

INSTRUMENTATION

All instruments were calibrated and maintained according to standard quality control practices and procedures. UV-Vis measurements were obtained on a Shimadzu BioSpec-Nano Micro-Volume UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). Mass measurements were taken on a Shimadzu AUW120D analytical balance. Activity measurements were obtained using a Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ). Biodistribution samples were

counted for activity for 1 min on a calibrated Perkin Elmer (Waltham, MA) Automatic Wizard Gamma Counter. Radiolabeling of the [^{64}Cu]-SarAr-Tz radioligand was monitored using silica-gel impregnated glass-fiber instant thin-layer chromatography paper (Pall Corp., East Hills, NY) and analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, D. C.).

HPLC: HPLC purifications (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in CH_3CN) were performed on a Shimadzu UFLC HPLC system equipped with a DGU-20A degasser, a SPD-M20A UV detector, two LC-20AP pump systems, a CBM-20A communication BUS module, and a FRC-10A fraction collector using a reversed-phase C_{18} XTerra® Preparative MS OBDTM column (10 μm, 19.2 mm × 250 mm; 10 mL/min) or a reversed-phase C_{18} semi-Prep Phenomenex® Jupiter column (5 μm, 10 mm × 250 mm; 2 mL/min). Analytical HPLC runs were performed using a reversed-phase C_{18} Phenomenex® Jupiter column (5 μm, 4.6 mm × 250 mm; 1 mL/min). All radio-HPLC analyses and purifications were performed using a Shimadzu HPLC equipped with a reversed-phase C_{18} column (Phenomenex Luna analytical 4.6 x 250 mm; 1 mL/min), 2 LC-10AT pumps, a SPD-M10AVP photodiode array detector, and a Bioscan Flow Counts radioactivity detector with a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 15 min.

High Resolution Mass Spectrometry: HRMS data were obtained on an Agilent 6550 QToF with a dual sprayer ESI source, coupled to an Agilent 1290 Infinity LC system. Samples were analyzed by either FIA (flow injection analysis) using a mobile phase of 50% acetonitrile in water (0.1% formic acid) with a flow rate of 0.4 mL/min; or LCMS with an Agilent Poroshell 120 SB-C18 column (2.7um, 2.1x50mm) at 45 degrees C, using a linear gradient of 5-95% acetonitrile in water (0.1% formic acid) with a flow rate of 0.4 mL/min. Mass spectra were obtained in either positive or negative mode, and acquired using the MassHunter Acquistiion Software (version B.05.01); then analyzed by MassHunter Qualitative Analysis (version B.06.00).

SYNTHESES

Synthesis of the TCO-Bearing Dendrimer

Synthesis of $G_{0.5}$PAMAM-SH

Reduction of the G_1-PAMAM cystamine-core dendrimer took place in an aqueous reaction buffer (50 mM HEPES, 10 mM EDTA, 2.5 mM TCEP). 375 µL of dendrimer (105 mM in ethanol) was added to 6 mL of buffer solution and let mix for 60 min at 25°C. Reaction proceeded quantitatively as confirmed by analytical C_{18} HPLC using a gradient of 2:98 MeCN:H_2O (both with 0.1% TFA) to 20:80 MeCN:H_2O over 50 minutes (t_R = 15.0 min). Reduced dendron confirmed by HPLC and HRMS (ESI) m/z: calcd for $C_{32}H_{67}N_{13}O_6S$, 761.5058; found, 761.5124.

Synthesis of $G_{0.5}$PAMAM-PEG_4-DBCO "DBCO-DEN-NH_2"

Without purification, 3 molar equivalents of DBCO-PEG_4-Maleimide (37.8 mg, 148 mM in DMSO, 674.75 g/mol) were added to the dendron mixture and allowed to mix for 24 hours at 25 °C. The reaction was purified by preparative C_{18} HPLC using a gradient of 20:80 MeCN:H_2O (both with 0.1% TFA) to 80:20 MeCN:H_2O over 20 minutes (t_R = 13.5 min). Lyophilization of HPLC eluent yielded the product as 22.97 mg of an orange, crystalline solid (MW = 1436.78 g/mol, 16.0 µmol, 40.6 %yield). HRMS (ESI) m/z: calcd for $C_{68}H_{109}N_{17}O_{15}S$, 1435.802; found, 1436.806.

Synthesis of $(TCO)_4$-$G_{0.5}$PAMAM-PEG_4-DBCO "DBCO-DEN-TCO"

$G_{0.5}$PAMAM-PEG_4-DBCO (6.5 mg, 4.5 µmol) was dissolved in DMSO to a final concentration of 1.2 mM. Eighty molar equivalents of triethylamine (36.7 mg, 360 µmol, 101.2 g/mol) were added to this solution, followed by 20 molar equivalents of TCO-NHS (24.2 mg, 90.0 µmol, 267 g/mol). The solution was allowed to react at 25 °C over 48 h and purified by preparative C_{18} HPLC using a gradient of 50:50 MeCN:H_2O (both without TFA) to 100:0 MeCN:H_2O over 30 minutes (t_R = 23.5 min). HPLC eluent was lyophilized, yielding the product as 7.59 mg of orange solid (MW = 2063.57, 3.68 µmol, 81.8 %yield). HRMS revealed that the maleimide underwent hydrolysis, however it has been demonstrated in literature that this affects neither the linkage nor the utility of the molecule.[3] HRMS (ESI) m/z: calcd for $C_{104}H_{159}N_{17}O_{24}S$, 2062.1465; found, 688.7231 $[M + 3H]^{3+}$.

Synthesis of ^{ss}huA33-DEN-TCO

Glycan Modification

HuA33 (10 mg, 11.92 mg/mL, in phosphate buffered saline [PBS], pH 7.4) was buffer exchanged into antibody preparation buffer (50 mM Bis-Tris, 100 mM NaCl, pH 6.0) using centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA). Following buffer exchange, a fraction of the antibody (5 mg in 125 µL antibody preparation buffer) was combined with Remove-iT™ EndoS tagged with a chitin-binding domain (35 µL, 200 U/µL) and 50 µL of the supplied G6 buffer (10x concentrated) and diluted to 500 µL in water. The mixture was placed on an agitating thermomixer at 37 °C and 450 rpm overnight. After 24 h, 175 µL of chitin magnetic bead solution (New England BioLabs, Ipswich, MA) were washed with tris-buffered saline (TBS) and mixed into the antibody-enzyme solution and placed on ice for 10 minutes. The chitin beads, now binding the EndoS enzyme, were separated magnetically and the remaining deglycosylated huA33 solution was concentrated using centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA).

GalNAz Modification

Deglycosylated huA33 (3.8 mg, 17.0 mg/mL in TBS) was then combined with UDP-GalNAz (5 µL of a 40 mM solution), $MnCl_2$ (2.5 µL of a 1 M solution), Tris buffer (26 µL of a 1 M solution (pH 7.0)), Gal-T(Y289L) [15 µL of 0.29 mg/mL in 50 mM Tris, 5 mM EDTA (pH 8.0)] and H_2O (19 µL) and was incubated at 30 °C overnight. The following day, GalNAz-modified antibodies were isolated using centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA) and buffer-exchanged into PBS (pH 7.4).

Dendrimer Ligation: ^{ss}huA33-DEN-TCO

The purified GalNAz-modified antibody was then combined with 120 µL $(TCO)_4$-$G_{0.5}$PAMAM-PEG_4-DBCO (5 mM in DMSO) and incubated at 25 °C overnight. The following day, the completed immunoconjugate, "^{ss}huA33-DEN-TCO", was purified via size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare) and concentrated using

centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA) with a final yield of 2.6 mg in 500 µL PBS. MALDI-ToF spectra revealed a mass-difference between conjugated and un-conjugated huA33 suggesting 1.7-2.0 TCO-bearing dendrimers per antibody.

Synthesis of ^{ss}huA33-DEN-NH$_2$

800 µg of GalNAz-modified antibody was prepared as described, and combined with 27 µL DBCO-DEN-NH$_2$ (2 mM in DMSO) and incubated at 25 °C overnight. The following day, the completed immunoconjugate, "^{ss}huA33-DEN-NH$_2$", was purified via size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare) and concentrated using centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA) with a final yield of 600 µg in 240 µL PBS. MALDI-ToF spectra revealed a mass-difference between conjugated and un-conjugated huA33 suggesting 2.0 ligands-per-antibody.

Synthesis of ^{ss}huA33-PEG$_{12}$-TCO

Synthesis of ^{ss}huA33-PEG$_{12}$-TCO was prepared as previously described.[4] In brief, huA33 antibody was modified by cleaving the terminal galactose residues from its heavy chain glycans using β-1-4-galactosidase. These were then replaced as described above with an azide-bearing galactose residue, GalNAz, by a promiscuous galactosyltransferase Gal-T(Y289L). Finally, TCO-PEG$_{12}$-DBCO was attached through strain-promoted azide-alkyne cycloaddition (SPAAC) ligation to the GalNAz residue and purified to yield the final product. MALDI-ToF confirmed the addition of 2 TCO ligands per mAb.

Synthesis of DFO-^{ss}huA33-DEN-TCO, DFO-^{ss}huA33-PEG$_{12}$-TCO, DFO-^{ss}huA33-DEN-NH$_2$, DFO-huA33, and DFO-huA33-EndoS

In order to study *in vivo* blood kinetics of the dendrimer-bearing immunoconjugates, a number of huA33 derivatives were radiolabeled with ^{89}Zr through the non-site-specific attachment of 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-(N-acetylhydroxylamino)- 6,11,17,22- tetraazaheptaeicosine] thiourea (p-SCN-Bn-DFO). The huA33 constructs modified were as follows: ^{ss}huA33-DEN-TCO, ^{ss}huA33-TCO, huA33, endoS-

deglycosylated huA33 (huA33-EndoS), and ^{ss}huA33-DEN-NH_2. A solution of 400 µg antibody in PBS (2.7 nmol, 2 g/L) was adjusted to pH 8.7 using Na_2CO_3 (0.1 M). Next, five molar equivalents of p-SCN-Bn-DFO (13.3 nmol, 2 mM in DMSO) were added and the reaction mixed for 1 hr at 37 °C. The conjugates were then purified by size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare) and concentrated using centrifugal filtration units with a 50,000 molecular weight cut-off (Amicon™ Ultra, Millipore Corp., Billerica, MA) with yields between 77-95%.

MALDI-ToF Mass Spectrometry

As a means by which to quantify the number of ligands per antibody, the immunoconjugates were analyzed by MALDI-ToF MS/MS using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH). 1 µL of each sample (1 mg/mL) was mixed with 1 µL of sinapic acid (10 mg/ml in 50% acetonitrile:water and 0.1% trifluoroacetic acid). 1 µL of the sample/matrix solution was spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH). Ions were analyzed in positive mode and external calibration was performed by use of a standard protein mixture (Bovine Serum Albumin).

SDS-PAGE Analysis of Antibody Conjugation

1 µg of antibody (2 µL of a 0.5 mg/mL stock) was combined with 18.5 µL H_2O, 3 µL 500 mM dithiothreitol (NuPAGE® 10X Sample Reducing Agent, Life Technologies), and 7.5 µL 4X electrophoresis buffer (NuPAGE® LDS Sample Buffer, Thermo Fisher, Eugene, OR). This mixture was then denatured by heating to 85 °C for 15 min using an agitating thermomixer. Subsequently, 20 µL of each sample was loaded alongside an appropriate molecular weight marker (Novex® Sharp Pre- Stained Protein Ladder, Life Technologies) onto a 1 mm, 10 well 4-12% Bis-Tris protein gel (Life Technologies) and run for ~5 h at 50 V in MOPS buffer. The gel was washed 3 times with H_2O, stained using SimplyBlue™ SafeStain (Life Technologies) for 1 h, and destained overnight in H_2O. The gel was then analyzed using an Odyssey CLx Imaging system (LI-COR Biosciences, Lincoln, NE).

Synthesis of Tz-SarAr

The ligand Tz-SarAr was synthesized as previously described.(*1*) In brief, a solution of Tz-NHS (5.0 mg; 0.013 mmol; 1.0 equiv.; 398.4 g/mol) in anhydrous dimethylformamide (400 µL) was added to a stirred solution of SarAr-Bn-NH_2 (5.4 mg; 0.013 mmol; 1.0 equiv.; 434.7 g/mol) in anhydrous dimethylformamide (200 µL) at room temperature, and the reaction solution was stirred in the dark for 2 h at room temperature. After dilution with water (1.8 mL), purification by HPLC (1.0 mL/min, 95:5 H_2O:CH_3CN to 20:80 H_2O:CH_3CN over 15 min) afforded Tz-SarAr (MW = 716.9; 3.9 mg; 42%) as a pink solid: t_R = 9.5 min. ^{1}H NMR (600 MHz, D_2O), δ, ppm: 10.25 (s, 1H), 8.31 (d, 2H), 7.46 (d, 2H), 7.22-7.27 (m, 4H), 4.39 (m, 2H), 4.24 (m, 3H), 2.46-3.95 (m, 24H), 2.41-2.44 (m, 4H), 1.83 (m, 2H). ^{13}C NMR (125 MHz, D_2O): δ = 21.7, 34.7, 42.5, 42.7, 47.4, 52.5, 55.9, 57.2, 58.8, 60.1, 116.2 (TFA), 127.7, 128.0, 128.5, 130.3, 135.4, 138.5, 143.5, 157.3, 162.9 (TFA), 166.3, 175.7, 175.8. ESI-MS(+): m/z = 717.6 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{36}H_{57}N_{14}O_2$: 717.4789; found: 717.4788.

Radiolabeling

Radiolabeling of DFO-mAb constructs with ^{89}Zr

^{89}Zr arrived as [^{89}Zr]Zr-oxalate in 1 M oxalic acid. An aliquot of 3 mCi (111 MBq) was diluted to 100 µL in oxalic acid (1 M). The pH was adjusted to 7.4 with Na_2CO_3 (1 M) and the solution then diluted to 500 µL in metal-free PBS (pH 7.4). 500 µCi aliquots were then combined with solutions containing 300 µg of huA33 constructs (DFO-^{ss}huA33-DEN-TCO, DFO-^{ss}huA33-TCO, DFO-huA33, DFO-huA33-EndoS, DFO-^{ss}huA33-DEN-NH_2) to a total volume of 500 µL and let mix at 25 °C for 1 h. Radiolabeling was verified as >98% RCY as determined by instant-thin layer chromatography using glass microfiber chromatography paper impregnated with silica gel (Varian Inc., Lake Forest, CA) as the stationary phase and 50 mM EDTA (pH 5.5) as the mobile phase. Finally, the radiolabeled constructs were purified by size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare) in PBS (pH 7.4).

Radiolabeling of Tz-SarAr with ^{64}Cu

Radiolabeling of Tz-SarAr with [^{64}Cu]$CuCl_2$ was carried out as previously described.(*1*) In brief, an NH_4OAc (0.1 M, pH 4.5) buffer solution containing Tz-SarAr (0.8 nmol) was mixed with 185 MBq (5 mCi) [^{64}Cu]$CuCl_2$ at 25 °C for 20 minutes. RadioHPLC confirmed >99% yield,

so radioligand was used without further purification. The solution was diluted in 0.9% sterile saline and adjusted with NaOH (5 M) to pH 7.4.

IN VITRO STUDIES

Saturation Binding Assay

Immunoreactivity of ^{ss}huA33-DEN-TCO and ^{ss}huA33-PEG$_{12}$-TCO conjugates was determined using a saturation binding assay with SW1222 cells expressing the GPA33 antigen. Briefly, 9 x 10^6 cells in 100 µL of PBS were aliquoted into Protein LoBind Eppendorf Tubes (Eppendorf AG, Hamburg, Germany) that had been pre-treated for 24 h with PBS + 1% fetal bovine serum. Concurrently, 150 µg of each mAb-TCO conjugate was separately mixed with [^{64}Cu]Cu-SarAr-Tz in PBS (pH 7.4) for 1 h at 25 °C, then purified by size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare) in PBS (pH 7.4). The resulting solution of "pre-clicked" [^{64}Cu]Cu-SarAr-mAb was diluted to 0.4 µg/mL and 2 ng aliquots were mixed with the microcentrifuge tubes containing SW1222 cells. The tubes were placed on ice for 1 h after which they were pelleted by centrifugation (4 min at 300 x g). The supernatant was then removed and set aside for counting. PBS with 1% FBS was added to the tubes, which were then re-pelleted. Supernatant was again set aside for counting and a second wash was performed. After washing, the activity in each of the tubes - cell pellet, supernatant, wash 1, and wash 2 - were counted separately and the immunoreactive fraction (taken here as percent bound) was calculated by dividing the counts in the cell pellet with the sum counts in the supernatant and washes. Data were obtained in quadruplicate for each construct.

Cell Culture

SW1222 human colorectal cancer cells, obtained from the Ludwig Institute of Cancer Research, were maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% inactivated fetal calf serum, 2.0 mM glutamine, 100 units/mL penicillin and 100 units/mL streptomycin. The cells were incubated at 37 °C in an environment containing 5% CO_2. Cells were passaged weekly and harvested using a mixture of 0.25% trypsin and 0.53 mM EDTA in Hank's Buffered Salt Solution without calcium or magnesium.

IN VIVO STUDIES

Xenograft Models

Female athymic nude mice (6-8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and allowed to acclimatize for approximately 1 week prior to inoculation. They were housed in ventilated cages and provided food and water as needed. They were anaesthetized using a mixture of 2% isofluorane (Baxter Healthcare, Deerfield, IL) and oxygen gas for 5 minutes before being moved to the table where isoflurane was maintained for the duration of procedure. Tumors were induced by the subcutaneous injection of $5x10^6$ SW1222 cells suspended 150 μL of a 1:1 mixture of cell media and Matrigel Matrix (Corning Life Sciences). The tumors reached the ideal size for imaging (~100-150 cm^3) after approximately 14 days.

Pretargeted PET Imaging Experiments

All pretargeted PET imaging experiments were performed on a Focus 120 microPET scanner (Siemens Healthcare Global). Female athymic nude mice (n = 3-5) bearing subcutaneous SW1222 xenografts on their right shoulder (9 days post-inoculation) were intravenously injected with 100 μg (0.66 nmol) of either ^{ss}huA33-DEN-TCO or ^{ss}huA33-PEG_{12}-TCO (150 μL in 0.9% sterile saline). After an accumulation interval of 120 hours they were then administered 16.7 MBq (450 μCi) of [^{64}Cu]Cu-SarAr-Tz (0.75 nmol). Approximately 5 minutes prior to imaging, mice were anaesthetized by inhalation of 2% isoflurane gas (Baxter Healthcare, Deerfield, IL) and oxygen gas mixture, and placed on the scanner bed. Isoflurane was maintained at 2% for the duration of the scan. Static scans were taken at prescribed time points (4 h and 24 h) following injection of radioligand for a 15 min. total scan time. An energy window of 350−700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier rebinning, and images were then reconstructed using a two-dimensional ordered subset expectation maximization (2DOSEM) algorithm (16 subsets, 4 iterations) into a 128 × 128 × 159 (0.78 × 0.78 × 0.80 mm) matrix. Image data was normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection. However, no attenuation, scatter, or partial-volume averaging corrections were applied. Activity concentrations (percentage of injected dose per gram of tissue

[%ID/g]) and maximum intensity projections were determined by conversion of the counting rates from the reconstructed images. All resulting PET images were analyzed using ASIPro VM software.

Pretargeted Biodistribution Experiments

Following PET imaging or blood half-life experiments (see above), mice were euthanized according to MSKCC Institutional Animal Care and Use Committee-approved protocols by inhalation of CO_2 gas. Organs were then harvested, allowed to air-dry for 5 minutes, then placed in pre-weighed tubes, weighed again and finally placed on a gamma counter. Counts were corrected to activity using a calibration curve generated from known standards of ^{64}Cu or ^{89}Zr. Activity was then decay-corrected and normalized to percent injected dose per gram. PET images were acquired at 24 h post-injection of ^{89}Zr-labeled immunoconjugates for a 10 min total scan time. Scan data were treated as described above.

Blood Serum Half-Life

Female athymic nude mice (n = 5) not bearing tumors were administered ~60 μCi (1.5 μCi/μg) of one of the following ^{89}Zr-labeled antibodies by tail-vein injection: DFO-^{ss}huA33-DEN-TCO, DFO-^{ss}huA33-PEG_{12}-TCO, DFO-huA33, DFO-huA33-EndoS, or DFO-^{ss}huA33-DEN-NH_2. Following a prescribed time after injection (2, 6, 24, 48, 72, 120, and 144 h), blood was collected from the tail vein contralateral to that originally injected with a glass capillary tube. Capillary tubes were placed in pre-weighed tubes, weighed, and counted for activity on a gamma counter. Activity was then decay-corrected and normalized to percent injected dose per gram of blood.

Calculations of Serum Half-life

Blood serum half-lives for each construct were calculated by fitting a bi-exponential decay curve to %ID/g and blood-draw time values. This accounted for a short serum half-life during the period immediately following injection (t_a), as well as a long serum half-life during the terminal phase of clearance (t_b). The program GraphPad Prism 7^{TM} was used to calculate both half-lives and their relative contributions to the overall half-life (in %). Both half-lives were weighted based on their relative contributions using the following formula:

$$t_{1/2} = \frac{\left(t_a * \%_{fast}\right) + t_b * \left(100 - \%_{fast}\right)}{100}$$

Values for antibody half-lives are summarized in the **Table S1**.

SUPPLEMENTAL FIGURES

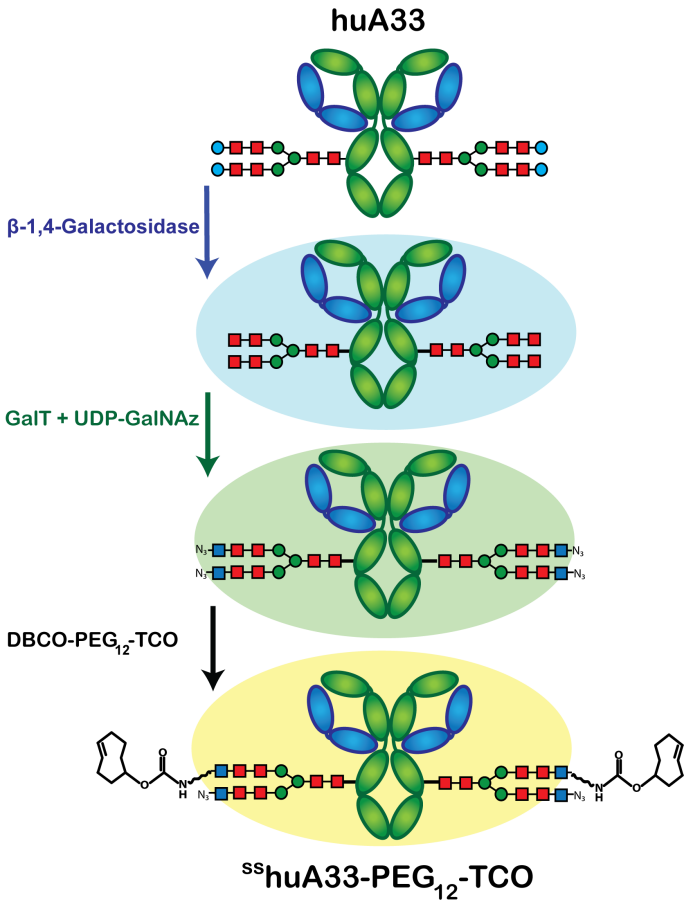

Figure S1. Chemoenzymatic strategy for the site-specific labeling of antibodies with β-1,4-galactosidase.

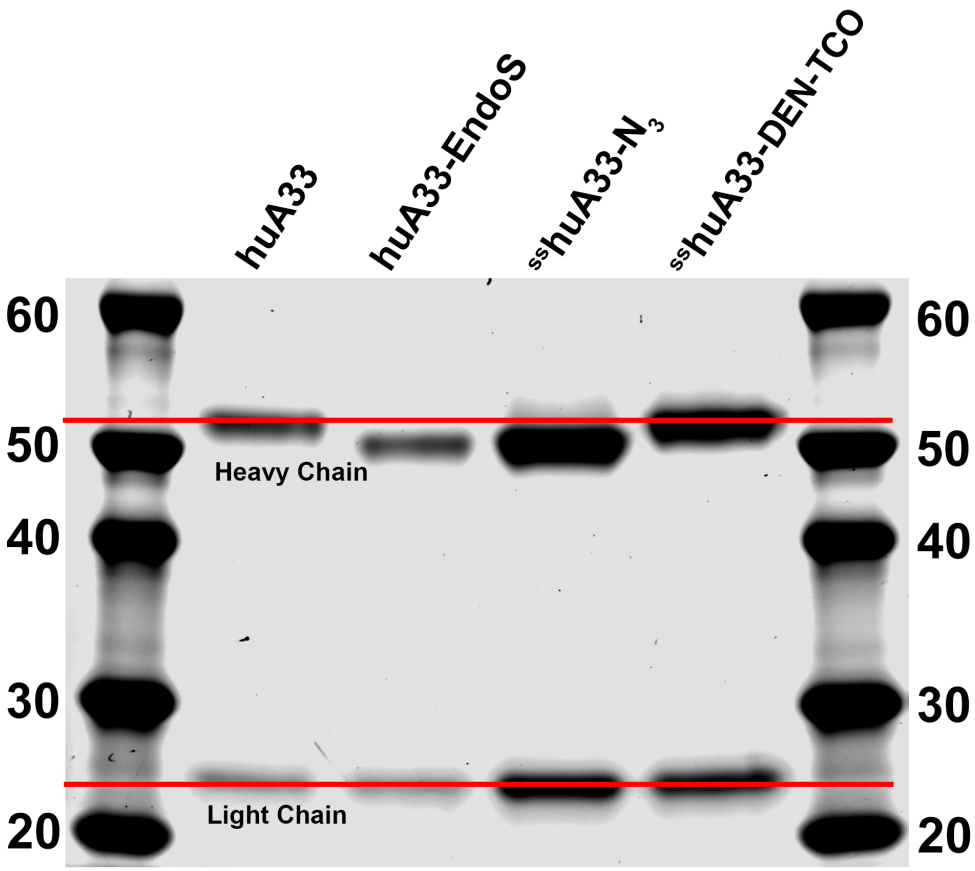

Figure S2. SDS-PAGE showing synthetic steps for making ^{ss}huA33-DEN-TCO. Top bands show shift in molecular weight of the antibody heavy chain as they are cleaved by EndoS and then bound by DBCO-DEN-TCO. The light chains (bottom) do not change in mass, indicating that modifications are specific to the heavy chain.

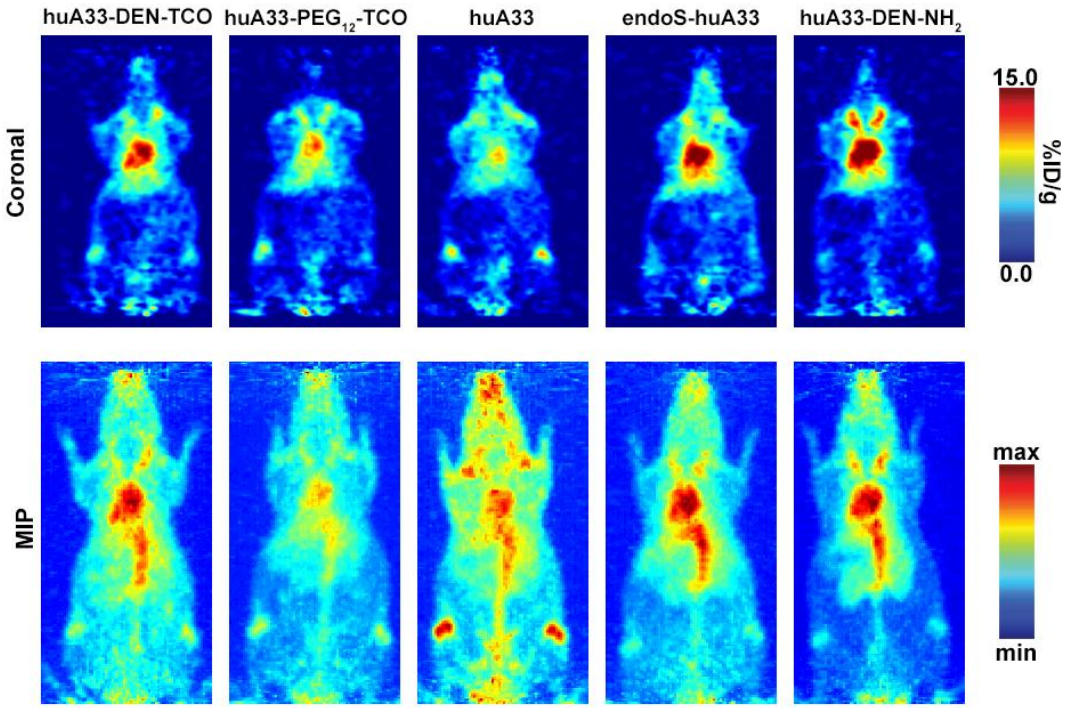

Figure S3. PET images of healthy athymic nude mice administered ^{89}Zr-DFO-labeled immunoconjugates. The images were collected 24 hours following the administration of the radiolabeled antibodies.

SUPPLEMENTAL TABLES

Table S1. Calculated serum half-lives of huA33 immunoconjugates.

Construct	$t_{1/2}$	t_b (slow) [hour]	t_a (slow) [hour]	% fast
^{ss}huA33-DEN-TCO	**77.7**	85.1	3.2	52.9
^{ss}huA33-PEG$_{12}$-TCO	**29.8**	33.0	3.0	54.7
huA33	**20.7**	37.1	7.0	53.9
huA33-EndoS	**58.9**	63.5	2.0	55.9
^{ss}huA33-DEN-NH$_2$	**33.9**	41.2	2.8	48.8

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